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Endophytic and Biological Control Potential of *Bacillus mojavensis* and Related Species

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The identity of a patented endophytic bacterium was established by 16S rRNA sequence analysis as a strain of *Bacillus mojavensis,* a recently erected species within one of the *B. subtilis* subgroups. This strain of *B. mojavensis* is antagonistic to the fungus *Fusarium moniliforme,* an endophytic mycotoxin-producing pathogen of maize and other plants. There are five other species within this subgroup: *Bacillus amyloliquefaciens, B. atrophaeus, B. licheniformis, Brevibacterium halotolerans, Paenibacillus lentimorbus,* and *P. popilliae.* The objectives of this research were to screen other isolates of *B. mojavensis, B. subtilis,* and the other closely related *Bacillus* species for endophytic colonizing capacity and to determine the *in vitro* antagonism to *F. moniliforme* in an effort to survey the distribution of these traits, which are desirable biological control qualities within the Bacillaceae. Antagonism was determined on nutrient agar, and endophytic colonization was established with maize plants following recovery of rifampin-resistant mutants generated from all strains used in the study. The study established that all 13 strains of *B. mojavensis,* isolated from major deserts of the world, endophytically colonized maize and were antagonists to *F. moniliforme.* The endophytic colonization of maize by *B. subtilis* and other species within this subgroup of the Bacillaceae varied, as did antagonism, to *F. moniliforme.* Thus, this study suggests that endophytic colonization is another characteristic of the species *B. mojavensis.* The endophytic habit and demonstrated antagonism to the test fungus indicate that isolates of this species might prove to be important biological control organisms where the endophytic habit is desired.

Key Words: Bacillus amyloliquefaciens; B. atrophaeus; B. licheniformis; B. mojavensis; B. subtilis;* biological control; bacterial antagonism; bacterial endophyte; fumonisins; *Fusarium moniliforme,* fungi; maize; mycotoxin; *Paenibacillus lentimorbus; P. popilliae; Zea mays.

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INTRODUCTION

Large numbers of fungal symbionts that are associated with plants are biotrophic mutualists, and several of these form endophytic associations (Bacon *et al.*, 1997; Bacon and De Battista, 1991; Keough *et al.*, 1996; White, 1994). Microbial endophytes actively colonize aboveground host tissues and establish long-term associations, actually lifelong natural associations, without doing substantive harm to the host. These associations are to be distinguished from transient visitors, usually dormant or latent infections, which form casual associations that do not survive long. Endophytic fungi are further distinguished by producing a variety of secondary metabolites *in planta* that impart toxicity to herbivores. Thus, fungal endophytes are capable of producing mycotoxins such as the fumonisins, beauvericin, fusaproliferin, fusaric acid, moniliformin (Leslie *et al.*, 1996; Marasas *et al.*, 1984; Shephard *et al.*, 1999), the ergot alkaloids (Bacon *et al.*, 1986), tremorgenic toxins (Munday-Finch *et al.*, 1995), and many other compounds that are biologically active (Bacon and Hinton, 1996a; Bush *et al.*, 1997; Porter, 1995; Siegel *et al.*, 1990; Stierle *et al.*, 1999). Endophytic fungi include not only the obligate species of *Neotyphodium, Balansia, Epichloe,* and *Myriogenospora,* but also facultative species in the genus *Fusarium*. These endophytic fungi are associated with thousands of plant hosts. Although some are very restricted and found associated with grasses, others are symbiotic with a wide variety of plant species, including both monocots and dicots.

Fusarium moniliforme Sheldon (synonym: *Fusarium verticillioides* (Sacc.) Nieberg; teleomorph *Gibberella moniliformis* Wineland) is one such facultative endophyte that has been isolated from at least 1100 hosts (Bacon *et al.*, 1996). This fungus produces five toxins. Although it is not known whether all five are produced during the endophytic colonizing stage, the fumonisins have been shown to be produced early by endophytic hyphae during maize seedling development (Bacon *et al.*, 2001). The fumonisin mycotoxins have been shown

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to be the cause of equine leucoencephalomalacia and porcine edema. *F. moniliforme*-infected maize is associated with animal and human esophageal cancer (Ross *et al.*, 1990; Marasas *et al.*, 1981). Recently, the fumonisins have been shown to be carcinogens (U.S. Department of Health and Human Services, 1999), and tolerance limits in maize are being considered (Troxell, 1996). Since *F. moniliforme* is endophytic in maize (Bacon and Hinton, 1996b) and almost universally associated with maize and maize products (Ayers *et al.*, 1989; Marasas *et al.*, 1984; Ross *et al.*, 1990; Schlechter *et al.*, 1998), it is very important that this fungus be controlled on this agriculturally important commodity. The endophytic habit of this toxic fungus makes it difficult to control with fungicides. However, biological controls may have the potential for control of endophytic fungi, and there are nontoxic endophytic microorganisms such as endophytic bacteria (Chanway, 1996; Hallmann *et al.*, 1997; Kirchhof *et al.*, 1997; Reinhold-Hurek and Hurek, 1998; Sturz *et al.*, 2000) that may control the endophytic hyphae of *F. moniliforme* and other fungi. The biological control strategy utilizing endophytic bacteria is expected to operate under the general mechanism of competitive exclusion, since bacterial growth within the intercellular spaces would preclude or reduce the growth by other microorganisms such as the intercellular hyphae of *F. moniliforme*.

Bacillus subtilis (Ehrenberg) Cohn is the oldest and the nomenclatural type species for the Bacillaceae and the genus *Bacillus*. This Gram-positive, spore-forming bacterium has proven safe over many years as a nonpathogenic species and is consumed in ton quantities in several human food preparations. The bacterium is widely distributed in nature and has been isolated from several botanical environments, primarily the soil, where it has been shown to have antibiotic properties and biological control potential. A strain of *B. subtilis*, RRC101, initially identified as *Enterobacter cloacae* and recently reported as a corn endophytic (Hinton and Bacon, 1995), is intercellular, nonpathogenic, an enhancer of plant growth, and a protector of plants against fungi. This strain was patented (Patent No. 5,994,117; ATCC 55732) as a biological control for diseases of maize caused by fungi (Bacon and Hinton, 1999). This isolate was subsequently found to belong to the closely related *B. subtilis*-like phenotype that was recently described as *Bacillus mojavensis* Roberts, Nakamura, & Cohan (Roberts *et al.*, 1994). *B. mojavensis* can be distinguished only by differences in whole-cell fatty acid composition, divergence in DNA sequence analysis, and resistance to genetic transformation between taxa within the *B. subtilis* group (Roberts *et al.*, 1994). The type and other strains of this species were isolated from soil samples from the Mojave Desert in California and other major deserts of the world, respectively (Table 1).

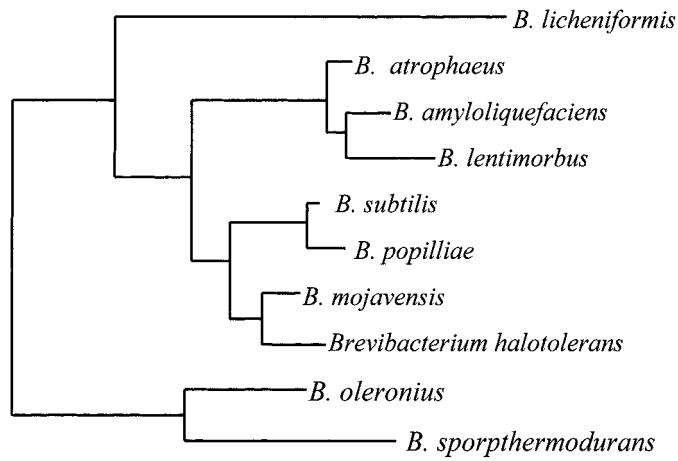

FIG. 1. Dendrogram of relationships for species within the *Bacillus subtilis* group established by 16S rRNA gene sequence analysis; horizontal distances correspond to levels of sequence divergence.

The patented strain of *B. mojavensis*, RRC101 (ATCC 55732), self-infects and endophytically colonizes maize seedlings after the topical application to seed, and the association which lasts for the duration of the growing season is mutualistic (Hinton and Bacon, 1995). These qualities suggest that endophytism in this strain is a biological requirement for survival in nature. Further, this endophytic colonizing ability might be characteristic of other strains of this newly erected species. The first objective of this study was to determine whether endophytic colonizations were characteristic of other strains of *B. mojavensis* and therefore a trait of the species. This patented strain is also antagonistic to most species of fungi, especially *F. moniliforme in vitro* and *in vivo* with a reduction in mycotoxin accumulation (Bacon *et al.*, 2001). Therefore, the second objective of this study was to determine whether other strains of *B. mojavensis* were also antagonistic to the fungus *F. moniliforme*. These two objectives were also extended to include other closely related species of the *B. subtilis* subgroup of organisms defined by Roberts *et al.* (1994) to further distinguish *B. mojavensis* within the Bacillaceae (Fig. 1).

MATERIAL AND METHODS

Bacterial Strains

Nutrient agar was used for routine laboratory maintenance of all bacteria. The bacteria used in this study are listed in Tables 1 and 2. These tables include those bacteria whose physiological, morphological, or metabolic phenotypes were identical, but whose DNA relatedness data (Fig. 1) and fatty acid analyses, according to Roberts *et al.* (1994), were different.

The strain RRC 101 is the patented endophytic strain (Patent No. 5,994,117) (Bacon and Hinton, 1999)

TABLE 1

Bacillus mojavensis Strains Used for Endophytic and Antagonistic Studies

Bacillus mojavensis strains[a]	Origin/source
RRC 101 (Patent 5,994,117, ATCC 55732)	Northern Italy, maize kernel
RRC 111	Transformant of RRC 101 (see text)
RRC 113 (UV12)	UV nonantagonistic mutant of RRC 111
RRC 114 (UV50)	UV nonantagonistic mutant of RRC 111
NRRL B-14698[T]	Mojave Desert, CA, soil
ATCC 51516	Mojave Desert, CA, soil
ATCC 51517	Gobi Desert, Inner Mongolia, China (Im-D-69 [NRRL B-14708])
NRRL B-14714	Sahara Desert, Nefta, Tunisia, soil
NRRL B-14711	Gobi Desert, Inner Mongolia, China, soil
NRRL B-14707	Gobi Desert, Inner Mongolia, China, soil
NRRL B-14709	Gobi Desert, Inner Mongolia, China, soil
NRRL B-14824	Sahara Desert, Nefta, Tunisia, soil
NRRL B-14818	Tumamoc Hill, AZ, soil
NRRL B-14700	Mojave Desert, CA, soil
NRRL B-14817	Tumamoc Hill, AZ, soil

[a] RRC, Russell Research Center culture collection, Athens, GA; NRRL, Northern Regional Research Laboratory, NCAUR, Peoria, IL; ATCC, American Type Culture Collection.

shown to confer disease protection and reduce the level of fumonisin (Bacon *et al.*, 2001) mycotoxin in maize seedlings and is used as the standard for comparison for all other *B. subtilis* strains and related species. This strain was patented under the name of *B. subtilis*, but 16S rRNA gene sequence analysis established this strain as *B. mojavensis* Roberts, Nakamura, and Cohan, a recently erected species that belongs to subgroup one of the *B. subtilis* complex of species (Fig. 1; Roberts *et al.*, 1994). Strain RRC 111 is an endophytic, antagonistic, auxotrophic transformant (*trpC2, thr5*) that was produced by transforming the nonendophytic, nonantagonistic, auxotroph (*trpC2, thr5*) *B. subtilis* BD 170 (ATCC 33608) with DNA from the endophytic antagonistic *B. mojavensis* RRC101 (Ganova-Raeva *et al.*, 1998). Two other strains, RRC 113 and RRC 114, were derived from RRC101 as random UV-resistant mutants, selected on the basis of being nonantagonistic to *F. moniliforme*.

Antagonism

Bacterial inocula were prepared from strains grown on nutrient agar for 7 to 10 days. The fungus used in this test was *F. moniliforme (=F. verticilloides)* RRC PATGUS, a strain rated as a strong pathogen of maize seedlings (Bacon *et al.*, 1992) and transformed (Yates *et al.*, 1999) with both the *gusA* reporter gene, which codes for â-glucoronidase (gus), and the *hph* gene, which codes for hygromycin resistance. Inoculum for the test fungus was prepared with 14-day-old cultures grown on potato dextrose agar plates (PDA; Difco, Detroit, MI). Control plates consisted of fungi or bacteria placed on nutrient agar plates alone as described above. A plug of *F. moniliforme* was placed on one side of a nutrient agar plate, and a loop of bacteria was streaked down the opposite side of the plate. The plates were incubated in the dark at 25–27°C until the fungi on the control plates had grown together. The first measure of antifungal activity from the bacteria was the size of the zone of inhibition formed between the radial growth on reverse, measured from the edge of the colony of *F. moniliforme* to the edge of a bacterial colony on the plate of nutrient agar. The second measure of antifungal activity, in the absence of an inhibition zone, was the appearance of hyphae in contact with the bacterial colony. The hyphal walls in contact with the bacterial colony were characterized by lysis and necrosis along the bacterial zone of contact with hyphae.

TABLE 2

Bacillus Species Used for Endophytic and Antagonistic Studies

Bacterial strains[a]	Origin/use
Bacillus amyloliquefaciens NRRL B-14393[T]	Soil, NRRL (from J. Fukomoto)
Bacillus atrophaeus NRRL NRS-213[T]	Colorado soil, NRRL (from N. R. Smith)
Bacillus subtilis BD170	T. Denny, Univ. Georgia, an autotrophy of ATCC 33608, D. Bubnau; see text
B. subtilis QKB105	T. Denny, Univ. Georgia, Athens
B. subtilis ATCC6051[T]	Marburgh strain, H. J. Conn (RF4738), the type
B. subtilis ATCC6633	N. R. Smith (NRS 231 by Kellerman), media testing
B. subtilis ATCC33608	D. Dubnau, transformation host (BD170)
B. subtilis ATCC49343	S. N. McDermott & T. F. Hartley (RF20336), antimicrobial test
B. subtilis ATCC55422	P. S. Patel (RF15007), inhibits bacteria
B. subtilis ATCC55614	P. G. Marrone (RF75329), plant disease control
B. subtilis ATCC55675	K. L. Brantly & R. R. Akins (RFRF177), plant transport enhancer
Paenibacillus lentimorbus NRRL B-2522[T]	L. K. Nakamura, NRRL
Paenibacillus lentimorbus NRRL B-2309[T]	L. K. Nakamura, NRRL

[a] NRRL, Northern Regional Research Laboratory, NCAUR, Peoria, IL; ATCC, American Type Culture Collection.

Effects of B. mojavensis on Plant Performance

The plant materials used to determine the effects of the bacteria on plant growth consisted of the maize cultivar 'Truckers Favorite,' a bush bean cultivar 'Blue Lake,' and a Durham wheat cultivar 'Ingot.' Seeds of these plants were subjected to a double-sterilization procedure (Bacon *et al.,* 1994) and then planted in a sterilized synthetic soil mix in 6-inch, plastic sterile pots. An aqueous suspension of washed bacterial inoculum (10^6 cfu/ml), prepared from a 48-h shake nutrient broth culture, was placed on the disinfected seeds, which were then air dried (approximately 12 h). Seed dried as described may be stored under aseptic conditions under refrigeration (4°C) for at least 6 weeks without germination being affected (data not shown). The inoculated seeds were planted in the sterile soil. All plants were grown under aseptic conditions in a plant growth light room at 32°C under a 16-h light (cool-white, high-output fluorescent tubes, an average of 254 μmol $\cdot$ $m^{-2} \cdot s^{-1}$) and an 8-h dark regime at 29°C for 14 to 21 days. Treated plants were harvested, washed, and separated into roots and stems. The soil growth medium was monitored throughout the experiment for sterility to both non-*B. mojavensis* bacteria and fungi. The lengths of roots and shoots were used to measure the effects of bacteria on plant growth. Results were reported as an average of six or more measurements, and all experiments were repeated at least twice.

Endophytic Colonization

All experiments to evaluate the ability of bacterial strains to endophytically colonize plants were performed with rifampin-resistant mutants. Spontaneous chromosomal rifampin resistant (Rif^r) mutants of each test strain were generated on nutrient agar containing 100 μg/ml of rifampin (Sigma Chemical Co.). A single rifampin-resistant colony was selected for each strain or species and streaked once more onto rifampin-amended nutrient agar. To determine the stability of mutants, each was followed through 10 serial passages on nutrient agar without rifampin. Rifampin resistance of each strain was confirmed following the serial passages by plating onto nutrient agar amended with rifampin (100 μg/ml). Since rifampin-resistant mutants may not be identical to the wild-type parent (Compeau *et al.,* 1988), all mutants were compared for desired traits. All mutants had identical growth rates and maintained their endophytic colonizing ability, but several lost the ability to antagonize the test fungus. All bacteria used to determine effects on the growth of plants were the wild types. The working stock cultures of mutants were maintained on nutrient agar containing 50 μg/ml rifampin and stored at 4°C until used. Stock cultures of all strains were stored on silica gel at −30°C.

The maize seedling assay (Bacon *et al.,* 1994), which was modified to include all host plants, was used as the test for endophytic colonization by the bacterial strains. The seeds were surface and internally sterilized before use (Bacon *et al.,* 1994). They were then rinsed in sterile distilled water, soaked in water for 4 h, heat treated in a water bath at 60°C for 5 min, and rinsed in sterile cool water. Bacterial inocula (0.01 ml, 2×10^6 cfu/ml) were prepared from the Rif^r mutants and placed on seeds that were then incubated on moist filter paper at 25°C for 48 h. These kernels were planted in a sterile synthetic soil mix (Redi-Earth; pH 5.5–6.0) in 6-inch plastic pots (thinned to 12 plants per pot). Plants were watered daily. Starting when plants were 2 weeks old, they were fertilized at weekly intervals with a liquid 20-20-20 (N-P-K) fertilizer containing micronutrients. Uninoculated seeds were used as controls and planted as described for the treatment groups. Companion treatment groups consisting of non-Rif^r mutants were also used in both preliminary experiments and in the Rif^r mutant recovery experiments to confirm that the mutation did not alter the endophytic effects of the parental strains. Experiments on the ability of RRC101 to colonize bean, oat, rye, and wheat plants were identical to those conducted on corn seeds when using disinfected seed of these plants, inoculated and grown as described.

Two- to 4-week-old plant material was used for recovery of bacteria, which is the standard time used to determine endophytic colonization by the patented strain and in planta mycotoxin accumulation (Bacon and Hinton, 1996b; Bacon *et al.,* 2001). This time period was established from experiments conducted with RRC 101, which is used as the standard endophytic strain that colonized seedlings following the topical application to kernels during this time period (Bacon and Hinton, 1996b). The plant material used for recovery of bacteria consisted of roots that were surface-disinfested with 1% chloramine-T for 30 min and shoots that were surfaced-disinfested with commercial bleach, full strength (5.25% sodium hypochlorite) for 5 min (Stone *et al.,* 2000). Roots disinfected with bleach would readily take up the sodium hypochlorite and become sterilized internally, yielding no bacteria. Disinfected plant parts were cut into smaller (0.5- to 1-cm) sections, placed on rifampin-amended nutrient agar medium, and incubated for 5–14 days at 25°C. The presence of bacteria in roots was used only as a tentative indicator of endophytic infection because the bacteria could colonize roots from broken roots and abrasions present on root surfaces and remain in these locations. Hence, bacteria found in roots might not represent true endophytic colonization. However, the recovery of the Rif^r mutants from leaves and stems plated on rifampin-amended nutrient agar was used as the indicator of endophytism. Antibiotic masking of Rif^r mutants was not a problem in these short-term

TABLE 3

Endophytic Colonization of Maize Plants and *in Vitro* Assay for Inhibition to *Fusarium moniliforme* by *Bacillus mojavensis*[a]

Bacillus mojavensis strains	Endophytic colonizer	Fungal antagonism
RRC 101	Y	+++
RRC 111	Y	++++
ATCC 51516	Y	C
ATCC 51517	Y	C
NRRL B-14714	Y	+
NRRL B-14711	Y	+
NRRL B-14707	Y	C
NRRL B-14709	Y	C
NRRL B-14698[T]	Y	C*
NRRL B-14818	Y	++
NRRL B-14817	Y	+
NRRL B-14700	Y	–
RRC 113 (uv-12)	Y	–
RRC 114 (uv-50)	Y	–

[a] Y, an endophyte; –, no inhibition; +, weak (<3 mm) *in vitro* inhibition; ++, moderate (>3–9 mm) *in vitro* inhibition; +++, strong (>9–18 mm) *in vitro* inhibition; ++++, very strong (>18 mm) *in vitro* inhibition; C, a contact inhibition (no zone of inhibition but dead hyphae of *F. moniliforme*); C*, very strong contact kill.

pot culture studies. The controls, which consisted of noninoculated sterilized seeds that were germinated and grown as described for the treatment groups, did not yield bacterial colonies when their surface-disinfected leaves and stems were plated on rifampin nutrient agar.

Microscopy

Microscopic examinations were performed on all bacterial-colonized plants that yielded bacteria from leaves. *In planta* visualization was accomplished with plant materials cut into thin sections by hand with a razor blade and stained first with 2,3,5-triphenyltetrazolium chloride stain (Patriquin and Dobereiner, 1978) and then followed by a second stain for 1 min in an aqueous solution of 0.1% aniline blue (Bacon and Hinton, 1997). Both stains were prepared as sterile solutions. The double-stained material was observed as a wet mount under oil at 100X. The bacterial cells were found in the intercellular spaces of plant tissue as stained, blue-purple, rod-shaped cells. If the whole bacteria-infected coleoptile from a 2- to 3-day-old seedling was subjected to this staining procedure, the region containing the bacteria was stained darkly, providing quick evidence of bacterial endophytic colonization that was visible to the unaided eye.

Data Analysis

All analyses were performed using software of the Microsoft Data Analysis System. Analysis of variance (ANOVA) was used to compare the biocontrol efficacy of endophytic strains and species on root and shoot growth, and treatment means were separated by Fisher's protected least significant difference (LSD). The significance of effects of endophytic bacterial treatments on growth characteristics was determined by the magnitude of the F value ($P = 0.05$).

RESULTS AND DISCUSSION

All Rif[r] mutant strains of *B. mojavensis* were isolated from the surface-disinfected roots, stem, and leaves of maize seedlings, indicating that these strains were endophytic (Table 3). However, the roots and stems were more heavily colonized by bacteria than were the leaves and the upper shoots of seedlings of the three plant species used. This distribution was true for all 13 *B. mojavensis* strains and mutants. The ability to establish endophytic associations with maize varied among the other species of bacteria (Table 4). The strain of *B. licheniformis* and both species of *Paenibacillus* were endophytic in maize but were characterized as noninhibitory to weakly inhibitory to *F. moniliforme*, respectively. Some strains of *B. subtilis*, the strains of *B. amyloliqufaciens* and *B. atrophaeus*, were isolated from roots but not from the above-ground portions of plants, indicating that these strains were not endophytic as defined in this paper. Their presence in root tissues probably reflected a very transient association, and we assume that they were randomly distributed within the damaged portions of roots formed dur-

TABLE 4

Endophytic Colonization of Maize Plants and *in Vitro* Assay for Inhibition of *Fusarium moniliforme* by Related *Bacillus* Species[a]

Bacillus subtilis & related species	Endophytic colonizer	*In vitro* inhibition
B. subtilis BD 170	N	–
B. subtilis QKB105	N	–
B. subtilis ATCC6051[T]	N	–
B. subtilis ATCC6633	N	–
B. subtilis ATCC3608	N	–
B. subtilis ATCC49343	N	–
B. subtilis ATCC55422	Y	++
B. subtilis ATCC55614	Y	+++
B. subtilis ATCC55675	N	–
B. atrophaeus[T]	N	+++
B. licheniformis[T]	Y	–
B. amyloliquefaciens[T]	N	C*
Paenibacillus lentimorbus[T]	Y	++
P. poppilliae[T]	Y	+

[a] Y, an endophyte; –, no inhibition; +, weak (<3 mm) *in vitro* inhibition; ++, moderate (>3–9 mm) *in vitro* inhibition; +++, strong (>9–18 mm) *in vitro* inhibition; ++++, very strong (>18 mm) *in vitro* inhibition; C, a contact inhibition (no zone of inhibition but dead hyphae of *F. moniliforme*); C*, very strong contact kill.

ing growth. However, two strains of *B. subtilis* were rated as endophytic (Table 4). A strain of *B. amyloliquefaciens* was reported as an endophyte, but the details of its isolation were not reported (Mari *et al.*, 1996). In addition to corn, *B. mojavensis* RRC101 was endophytic in beans, oats, barley, rye, and wheat (data not shown).

Eleven of the 13 strains of *B. mojavensis* showed *in vitro* inhibition to *F. moniliforme,* although some were more inhibitory than others (Table 3). Observations on the inhibitory response suggested that not all strains produced the same inhibitory substance. For example, some strains caused fungal lysis upon contact with hyphae, which eventually resulted in lysis of the entire fungal colony. This type of inhibition was referred to as contact inhibition (Table 3). Other bacteria produced a diffusable inhibitory substance into the medium that produced necrotic areas in hyphae along the edge of a colony. Strain NRRL B-14698, the type, showed very strong antagonism via contact inhibition. Contact inhibition is very different from the inhibition expressed by the biocontrol, patented isolate RRC 101, which was the diffusion type. The mutant RRC 111 produced a greater inhibitory response than the parent RRC 101. Two strains, NRRL B-14817 and NRRL B-14700, showed very weak *in vitro* inhibition. The two UV mutants, RRC 113 (UV12) and RRC 114 (UV50), were selected (unpublished data) on the basis of being noninhibitory to fungi but were derived from the biocontrol bacterium RRC 101. These two nonantagonistic mutants are intended for use in experiments designed to determine the *in planta* inhibitor production and its role in control of fungal growth. However, strain NRRL B-14700 was the only natural isolate that was not antagonistic to the fungus.

The variation in both the potency and the type of antagonism to the fungus might reflect either the amount or the types of inhibitory substances produced, which also might be unstable or poorly diffused into the agar. Alternatively, each strain of *B. mojavensis* may have membranes that are differentially permeable to the inhibitor, thereby restricting its diffusion into the medium. Another possibility is that the fungus is inhibited by nonantibiotic mechanisms. However, the differences in the appearance in the hyphae due to either contact or diffusional inhibition suggest that there are probably more than one inhibitor produced by strains. We have no information on the chemical identity of substances produced by any of these isolates, but further studies should provide insight into the chemical nature of the antibiotic and its significance, if any, in the biological control performance observed in these studies. Finally, the *in vitro* antagonism was measured on nutrient agar, but similar data were obtained with modifications to this medium (data not shown) or with an entirely different medium (Ganova-Raeva *et al.,* 1998). It is expected that the production of an antibiotic substance by strains and species might be media specific. Thus, the nonantagonism observed by the coculturing of bacteria with fungi might reflect a lack of nutritional requirements for this effect.

Seven of the nine *B. subtilis* strains, including the type strain, were noninhibitory to the fungus (Table 4). These strains included several that were used as control for fungi. The two strains of *B. subtilis* that were scored with moderate to strong inhibition to *F. moniliforme* were ATCC55422 and ATCC55614, respectively, which are two patented strains used to inhibit microorganisms (Table 2). These two strains of *B. subtilis* were also unique from the other strains in being endophytic (Table 4). The other species that was negative for *in vitro* inhibition to *F. moniliforme* was *B. licheniformis*. The rest of the species within the Bacillaceae varied in their *in vitro* inhibitory reaction to the fungus. The type strain for *B. amyloliquefaciens* Priest (*ex* Fuk.) NRRL B-14393 showed the strongest inhibition, even though the form of inhibition was contact. In this regard, the inhibition was similar to that produced by some strains of *B. mojavensis,* especially the type strain NRRL B-14698. Another strain of *B. amyloliquefaciens* produced an antibiotic substance that was an effective antagonist to *Botrytis cinera* (Mari *et al.*, 1996).

All *B. mojavensis*-infected plants were symptomless, and their growth either was comparable or exceeded the growth of noninfected control seedlings (Fig. 2). The growth response produced by the patented *B. mojavensis* strain was also reflected in increased root growth typified by corn and beans (Fig. 3). There was a 70% average increase in root and shoot growth among all strains of *B. mojavensis*-infected plants over the noninfected control group (Table 5). All strains of *B. mojavensis* were similar to the biocontrol strain RRC101, as they colonized germinating seedlings following the topical application to corn, wheat, and bean seed.

Compared to our knowledge of fungal endophytes, relatively little is known about bacterial endophytes. *B. subtilis* and other members of the Bacillaceae are not known to be plant pathogens. We now report for the first time that *B. mojavensis* is not a plant pathogen in maize and that this species can colonize beans, rye, oats, barley, and wheat. This bacterium is not host specific and is capable of internally colonizing a wide variety of plant material. Thus, *B. mojavensis* may be further distinguished from *B. subtilis* in being endophytic, which might prove to be characteristic of this new species when additional strains are isolated and tested. Although variable, most strains of this species show *in vitro* antagonism to *F. moniliforme*. In addition to this fungus, the biocontrol isolate RRC101 is antagonistic to *Alternaria alternata, Cladosporium herbarum, Colletotrichum graminicola, Diplodia zeae, Hel-*

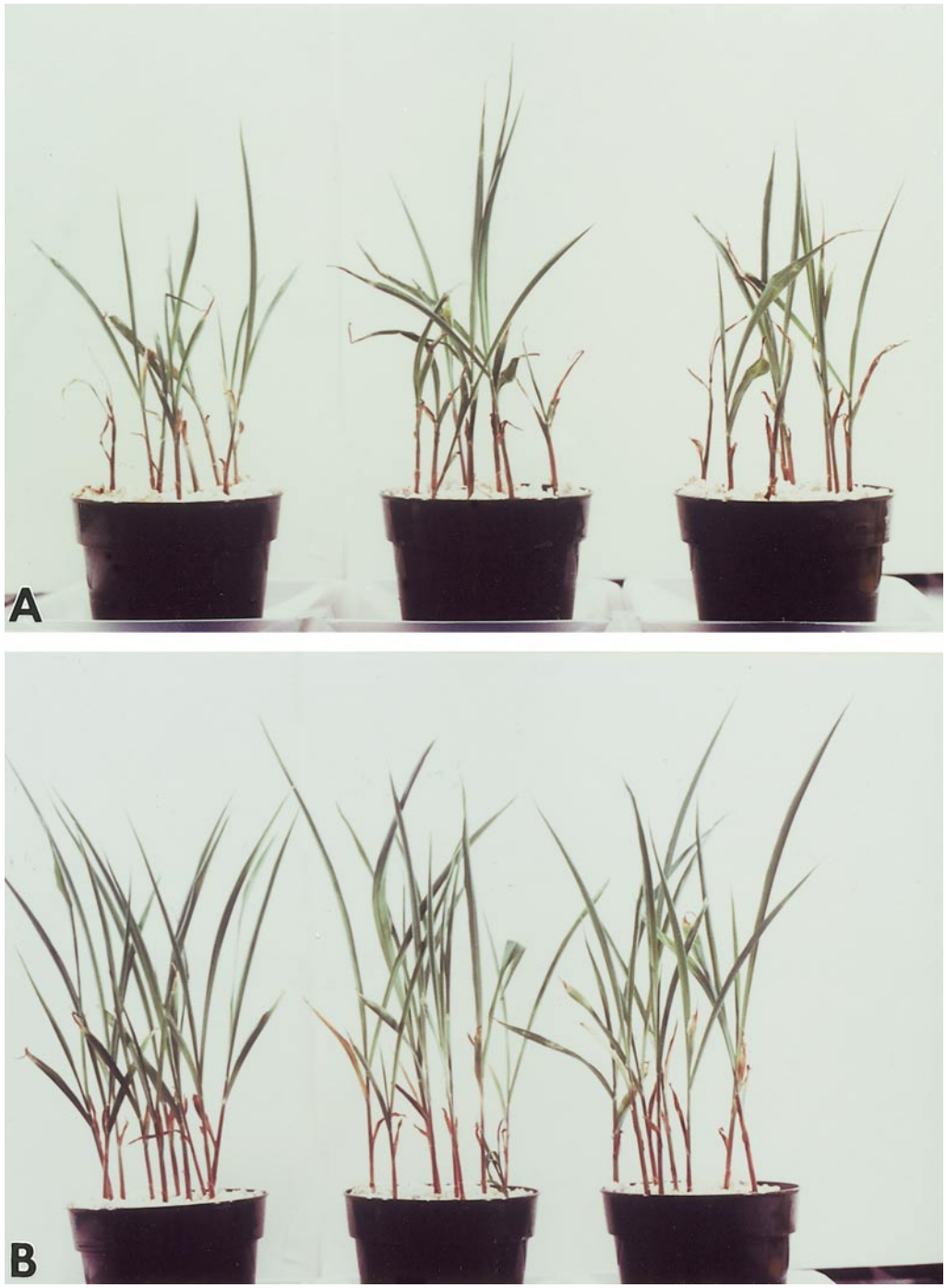

FIG. 2. Effects of *Bacillus mojavensis* RRC 101 on 3-week-old corn seedling growth. (A) Plants not infected with the biocontrol bacterium; (B) *B. mojavensis*-infected seedlings.

minthosporium carborum, *Penicillium chrysogeum*, *Phythium* sp., *Rhizoctonia solani*, *Aspergillus flavus*, and *A. parasiticus* (Bacon *et al.*, 2000). We do not have data on the antagonism of all *B. mojavensis* strains to these fungi, but the indications from this study are that other types of inhibitors are probably produced *in vitro*, suggesting that the toxicity to other organisms might be a general mechanism for all isolates.

The *in planta* location of all *B. mojavensis* strains reported here is intercellular. *B. mojavensis* has never been observed within plant cells (Fig. 4), including the xylem (Bacon and Hinton, 1966), which is characteristic of most bacterial and fungal endophytes. Many different bacteria have been isolated from surface-sterilized tissues of monocots and dicots, from crop and noncrop plants, and from herbaceous and woody plants

FIG. 3. Effects of *Bacillus mojavensis* on root growth, numbers, and size of lateral roots. (A1) *B. mojavensis*-infected corn seedlings roots; (A2) control noninfected corn seedling roots; (B1) control noninfected bush bean seedling roots; (B2) *B. mojavensis*-infected bush bean seedling roots.

(Bell *et al.*, 1995; Hollman *et al.*, 1997; Bent and Chanway, 1998; Shishido *et al.*, 1999). In the most reliable studies, bacteria have been isolated from sweet corn (Chelius and Triplett, 2000; Fisher *et al.*, 1992), cotton (McInroy and Kloepper, 1995; Quadt-Hallmann *et al.*, 1997), and other crops (Barraquio *et al.*, 1997; Bell *et al.*, 1995; Dong *et al.*, 1994; Sturz *et al.*, 1998). Both Gram-negative and -positive bacteria (including *B. subtilis*) have routinely been isolated from maize and other plants (Fisher *et al.*, 1992; McInroy and Kloepper, 1995; Palus *et al.*, 1996). Bacteria can be found in all plant tissues; they are generally in the intercellular spaces but have also been observed to be intracellular (especially within the xylem). Total internal bacterial populations are surprisingly high, ranging from 10^3 to 10^5 cells per gram fresh weight, and are usually higher in roots than in stems (Hallmann *et al.*, 1997).

It is important to note that the definition of a bacterial endophyte as used in this work is very restrictive, is based on careful analyses, and is an extension of that used for fungal endophytes (Stone *et al.*, 2000). This definition would exclude those bacteria that are transient colonizers found only in restricted areas of roots. Such organisms are epiphytic or epibiotic and are not considered here as true endophytic bacteria. Thus, it is uncertain whether numerous reports of endophytic bacteria have actually been subjected to experimental scrutiny to establish the diversity and nature of the endophytic habit and any possible benefits as those used here. The stringent definition of bacterial endophytes used in this work might reduce the number of bacterial endophytes considerably.

All nonpatented strains of *B. mojavensis* reported and used in this study were isolated from desert soils (Roberts *et al.*, 1994), which suggests that this bacterium is an endophyte of cacti and related plants. However, the isolation of the biocontrol patented strain from a sample of maize kernels indicated that the distribution might be wider than a desert niche. Further, other strains of *B. subtilis* isolated from plants as

TABLE 5

Effects of *Bacillus mojavensis* Strains on Maize Kernel Germination and Seedling Growth

B. mojavensis strains	% Germination[a]	Seedling growth, cm	
		Roots	Shoots
RRC 101	80a[b]	20.9b	22.2b
RRC 111	100b	20.9b	26.0c
ATCC 51516	80a	22.1b	21.0b
ATCC 51517	90c	21.1b	20.8b
NRRL B-14714	95bc	21.5b	21.2b
NRRL B-14817	100b	19.3b	23.7b
NRRL B-14711	90c	16.2c	22.7b
NRRL B-14707	60d	19.5ab	24.9bc
NRRL B-14709	95bc	14.6ac	18.2a
NRRL B-14824	95bc	20.5b	27.1c
NRRL B-14698	95bc	17.0c	24.2b
NRRL B-14818	90c	19.9b	22.5b
NRRL B-14700	90c	18.7b	21.8b

[a] Mean percentage germination; 20 seeds per pot, each pot replicated three times, and each experiment repeated twice.

[b] Mean values within a column followed by the same letter are not significantly different from a nontreated control according to the least significant difference test at $P = 0.05$.

endophytes may well be valid species of *B. mojavensis* when subjected to molecular analysis such as 16S rRNA sequence information, which is the basis for defining this species (Roberts *et al.*, 1994) and classifying bacteria (Weisburg *et al.*, 1991; Woese, 1987), rather than morphological and metabolic characteristics. In fact, there are no reported physiological or biochemical characteristics that would identify cryptic species within the section (Logan and Berkeley, 1984; Priest, 1993; Roberts *et al.*, 1994). The endophytic colonization of maize by the two strains of *B. subtilis* identified in this study suggests that these two might well be additional cryptic species or indeed, *B. mojavensis*. In either case, the distinction can be achieved by 16S rRNA sequences analysis (Roberts *et al.*, 1994).

Strains of *B. mojavensis* should make excellent agents for biological control of diseases, as suggested here, or insect pests (Sturz *et al.*, 2000). The endophytic location, the possible variations in secondary metabolites, i.e., fungal inhibitors, and the natural harsh desert environment reported for most isolates of this endophytic species should make it more persistent and reliable than common biological control agents that function in the harsh environment on the plant (usually root) exterior. While our experiments were not designed to determine the mechanism of action, the *in planta* method of disease control by endophytic bacteria may operate through either niche exclusion and preemptive competitive exclusion (Thomashow and Weller, 1996) or direct antagonism via antibiotic production. Non-antibiotic-producing endophytic bacteria may offer an advantage over the antibiotic-producing strains in terms of human and agricultural acceptance. We have *in vitro* evidence that there are producing and nonproducing antibiotically strains of *B. mojavensis*, but all may be negative for antibiotic production *in planta*. Because of their intimate association with plants, *B. mojavensis* endophytes might also improve general plant health by producing beneficial compounds. Indeed, the biocontrol isolate reported here improves the growth and greening of seedlings early in their life and reduces the accumulation of the fumonisin class of mycotoxins produced by *F. moniliforme* (Bacon *et al.*, 2001). While this might not hold true for all strains of *B. mojavensis*, other strains could offer unique beneficial traits and protective roles against other organisms in plants.

This study identifies a group of bacteria that may have the utility as biological controls for plant diseases and conferring other beneficial traits to plants. One can envision how molecular modifications of bacterial endophytes might be used to improve the nutritional value of their hosts when used as animal feed or to extend the range of compounds that could be detoxified during phytoremediation. We propose that such surrogately transformed plants might be easier to manipulate and could be more attractive alternatives to transgenic plants. We submit that isolates of *B. mojavensis* are favorable candidates for these important plant-enhancing characteristics.

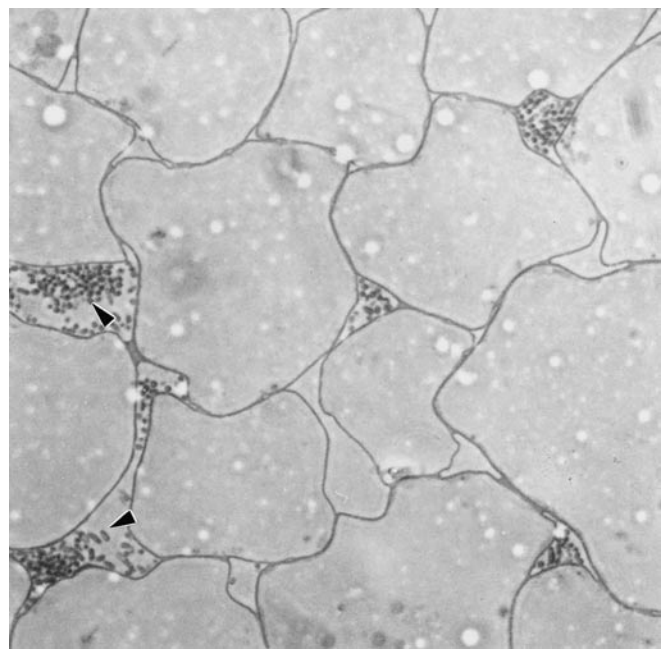

FIG. 4. Light micrograph (100X) of corn plant showing the distribution of bacterial cells of *Bacillus mojavensis* within the intercellular spaces of tissue (arrows).

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REFERENCES

Ayers, J. E., Nelson, P. E., and Krause, R. A. 1989. Fungi associated with corn stalk rot in Pennsylvania in 1970 and 1971. *Plant Dis. Reporter* **56,** 836–839.

Bacon, C. W., Bennett, R. M., Hinton, D. M., and Voss, K. A. 1992. Scanning electron microscopy of *Fusarium moniliforme* within asymptomatic corn kernels and kernels associated with equine leukoencephalomalacia. *Plant Dis.* **76,** 144–148.

Bacon, C. W., and De Battista, J. 1991. Endophytic fungi of grasses. *In* "Advances in Applied Mycology" (D. K. Arora, Ed.), pp. 231–256. Dekker, New York.

Bacon, C. W., and Hinton, D. M. 1996a. Fusaric acid and pathogenic interactions of corn and non-corn isolates of *Fusarium moniliforme,* a nonobligate pathogen of corn. *Adv. Exp. Med. Biol.* **392,** 175–191.

Bacon, C. W., and Hinton, D. M. 1996b. Symptomless endophytic colonization of maize by *Fusarium moniliforme. Can. J. Bot.* **74,** 1195–1202.

Bacon, C. W., and Hinton, D. M. 1997. Isolation and culture of endophytic bacteria and fungi. *In* "A Manual of Environmental Microbiology" (G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach, and M. V. Walter, Eds.), pp. 413–421. ASM Press, Washington, DC.

Bacon, C. W., and Hinton, D. M. 1999. "Use of *Bacillus subtilis* as an Endophyte for the Control of Diseases Caused by Fungi." US Patent & Trademark Office, November 30, 1999.

Bacon, C. W., Hinton, D. M., and Richardson, M. D. 1994. A corn seedling test for resistance to *Fusarium moniliforme. Plant Dis.* **78,** 302–305.

Bacon, C. W., Lyons, P. C., Porter, J. K., and Robbins, J. D. 1986. Ergot toxicity from endophyte-infected grasses: A review. *Agron. J.* **78,** 106–116.

Bacon, C. W., Porter, J. K., Norred, W. P., and Leslie, J. F. 1996. Production of fusaric acid by *Fusarium* species. *Appl. Environ. Microbiol.* **62,** 4039–4043.

Bacon, C. W., Richardson, M. D., and White, J. F., Jr. 1997. Modification and uses of endophyte-enhanced turfgrasses: A role for molecular technology. *Crop Sci.* **37,** 1415–1425.

Bacon, C. W., Yates, I. E., Hinton, D. M., and Meredith, F. 2001. Biological control of *Fusarium moniliforme* in maize. *Environ. Health Perspect.* **109,** 325–332.

Barraquio, W. L., Revilla, L., and Ladha, J. K. 1997. Isolation of endophytic diazotrophic bacteria from wetland rice. *Plant Soil* **194,** 15–24.

Bell, C. R., Dickie, G. A., Harvey, W. L. G., and Chan, J. W. Y. F. 1995. Endophytic bacteria in grapevine. *Can. J. Microbiol.* **41,** 46–53.

Bent, E., and Chanway, C. P. 1998. The growth-promoting effects of a bacterial endophyte on lodgepole pine are partially inhibited by the presence of other rhizobacteria. *Can. J. Microbiol.* **44,** 980–988.

Bush, L. P., Wilkinson, H. H., and Schardl, C. L. 1997. Bioprotective alkaloids of grass–fungal endophyte symbioses. *Plant Physiol.* **114,** 1–7.

Chanway, C. P. 1996. Endophytes: They're not just fungi. *Can. J. Bot.* **74,** 321–322.

Chelius, M. K., and Triplett, E. W. 2000. *Dyadobacter fermentans* gen. nov., sp nov., a novel Gram-negative bacterium isolated from surface-sterilized *Zea mays* stems. *Int. J. Syst. Bacteriol.* **50,** 751–758.

Compeau, G., Al-Chi, B. J., Platsouka, E., and Levy, S. B. 1988. Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. *Appl. Environ. Microbiol.* **54,** 2432–2438.

Dong, Z., Canny, M. J., McCully, M. E., Roboredo, M. R., Cabadilla, C. F., Ortega, E., and Rodés, R. 1994. A nitrogen-fixing endophyte of sugarcane stems: A new role for the apoplast. *Plant Physiol.* **105,** 1139–1147.

Fisher, P. J., Petrini, O., and Scott, H. M. L. 1992. The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). *New Phytol.* **122,** 299–305.

Ganova-Raeva, L. M., Denny, T. P., Boncheva, M., and Bacon, C. W. 1998. A novel *Bacillus subtilis* endophyte from corn with strong antifungal antivity. *Microbiol Abstracts* **1998,** 447. [Abstract]

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., and Kloepper, J. W. 1997. Bacterial endophytes in agricultural crops. *Can. J. Microbiol.* **43,** 895–914.

Hinton, D. M., and Bacon, C. W. 1995. *Enterobacter cloacae* is an endophytic symbiont of corn. *Mycopathologia* **129,** 117–125.

Keough, R. G., Tapper, B. A., and Fletcher, R. H. 1996. Distributions of the fungal endophyte *Acremonium lolii,* and of the alkaloids lolitrem B and peramine, within perennial ryegrass. *N. Z. J. Agric. Res.* **39,** 121–127.

Kirchhof, G., Reis, V. M., Baldani, J. I., Eckert, B., Döbereiner, J., and Hartmann, A. 1997. Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. *Plant Soil* **194,** 45–55.

Leslie, J. F., Marasas, W. F. O., Shephard, G. S., Sydenham, E. W., Stockenström, S., and Thiel, P. G. 1996. Duckling toxicity and the production of fumonisin and moniliformin by isolates in the A and F mating populations of *Gibberella fujikuroi (Fusarium moniliforme). Appl. Environ. Microbiol.* **62,** 1182–1187.

Logan, N. A., and Berkeley, C. W. 1984. Identification of *Bacillus* strains using the API system. *J. Gen. Microbiol.* **130,** 1871–1882.

Marasas, W. F. O., Nelson, P. E., and Toussoun, T. A. 1984. "Toxigenic *Fusarium* Species." Pennsylvania State Univ. Press, University Park, PA.

Marasas, W. F. O., Wehner, F. C., van Rensburg, S. K., and van Schlkwyk, D. J. 1981. Mycoflora of corn produced in human esophageal cancer areas in Transkei. *Phytopathology* **71,** 792–796.

Mari, M., Guizzardi, M., and Pratella, G. C. 1996. Biological control of gray mold in pears by antagonistic bacteria. *Biol. Control* **7,** 30–37.

McInroy, J. A., and Kloepper, J. W. 1995. Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant Soil* **173,** 337–342.

Munday-Finch, S. C., Miles, C. O., Wilkins, A. L., and Hawkes, A. D. 1995. Isolation and structure elucidation of lolitrem A, a tremorgenic mycotoxin from perennial ryegrass infected with *Acremonium lolii. J. Agric. Food Chem.* **43,** 1283–1288.

Palus, J. A., Borneman, J., Ludden, P. W., and Triplett, E. W. 1996. A diazotrophic bacterial endophyte isolated from stems of *Zea mays* L and *Zea luxurians* Iltis and Doebley. *Plant Soil* **186,** 135–142.

Patriquin, D. G., and Dobereiner, J. 1978. Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. *Can. J. Microbiol.* **24,** 734–742.

Porter, J. K. 1995. Analysis of endophyte toxins: Fescue and other grasses toxic to livestock. *J. Anim. Sci.* **73,** 871–880.

Priest, F. G. 1993. Systematics and ecology of *Bacillus*. *In* "*Bacillus subtilis* and Other Gram-Positive Bacteria" (A. L. Sonenshein, J. A. Hoch, and R. Losick, Eds.), pp. 3–16. Am. Soc. Microbiol., Washington, DC.

Quadt-Hallmann, A., Benhamou, N., and Kloepper, J. W. 1997. Bacterial endophytes in cotton: Mechanisms of entering the plant. *Can. J. Microbiol.* **43,** 577–582.

Reinhold-Hurek, B., and Hurek, T. 1998. Life in grasses: Diazotrophic endophytes. *Trends Microbiol.* **6,** 139–144.

Roberts, M. S., Nakamura, L. K., and Cohan, F. M. 1994. *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. *Int. J. Syst. Bacteriol.* **44,** 256–264.

Ross, P. F., Nelson, P. E., Richard, J. L., Osweiler, G. D., Rice, L. G., Plattner, R. D., and Wilson, T. M. 1990. Production of fumonisins by *Fusarium moniliforme* and *Fusarium proliferatum* isolates associated with equine leukoencephalomalacia and a pulmonary edema syndrome in swine. *Appl. Environ. Microbiol.* **56,** 3225–3226.

Schlechter, M., Marasas, W. F. O., Sydenham, E. W., Stockenström, S., Vismer, H. F., and Rheeder, J. P. 1998. Incidence of *Fusarium moniliforme* and fumonisins in commercial maize products, intended for human consumption, obtained from retail outlets in the United States and South Africa. *S. Afr. J. Sci.* **94,** 185–187.

Shephard, G. S., Sewram, V., Nieuwoudt, T. W., Marasas, W. F. O., and Ritieni, A. 1999. Production of the mycotoxins fusaproliferin and beauvericin by South African isolates in the *Fusarium* section *Liseola*. *J. Agric. Food Chem.* **47,** 5111–5115.

Shishido, M., Breuil, C., and Chanway, C. P. 1999. Endophytic colonization of spruce by plant growth-promoting rhizobacteria. *FEMS Microbiol. Ecol.* **29,** 191–196.

Siegel, M. R., Latch, G. C. M., Bush, L. P., Fannin, F. F., Rowan, D. D., Tapper, B. A., Bacon, C. W., and Johnson, M. C. 1990. Fungal endophyte-infected grasses: Alkaloid accumulation and aphid response. *J. Chem. Ecol.* **16,** 3301–3315.

Stierle, A. A., Stierle, D. B., and Bugni, T. 1999. Sequoiatones A and B: Novel antitumor metabolites isolated from a redwood endophyte. *J. Org. Chem.* **64,** 5479–5484.

Stone, J. K., Bacon, C. W., and White, J. F., Jr. 2000. An overview of endophytic microbes: Endophytism defined. *In* "Microbial Endophytes" (C. W. Bacon and J. F. White, Jr., Eds.), pp. 3–30. Dekker, New York.

Sturz, A. V., Christie, B. R., and Matheson, B. G. 1998. Associations of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. *Can. J. Microbiol.* **44,** 162–167.

Sturz, A. V., Christie, B. R., and Nowak, J. 2000. Bacterial endophytes: Potential role in developing sustainable systems of crop production. *Crit. Rev. Plant Sci.* **19,** 1–30.

Thomashow, L. S., and Weller, D. M. 1996. Current concepts in the use of introduced bacteria for biological disease control: Mechanisms and antifungal metabolites. *In* "Plant–Microbe Interactions" (G. Stacey and N. T. Keen, Eds.), pp. 187–235. Chapman & Hall, New York.

Troxell, T. C. 1996. Regulatory aspects of fumonisins in the United States. *Adv. Exp. Med. Biol.* **392,** 355–361.

U. S. Department of Health and Human Services. 1999. "Toxicology and Carcinogenesis Studies of Fumonisin B1 in F344/n Rats and B6C3F1 Mice." National Institutes of Health, Publication No. 99-3955, Bethesda, MD.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. 1991. Ribosomal DNA amplification for phylogenetic studies. *J. Bacteriol.* **173,** 697–703.

White, J. F. Jr. 1994. Taxonomic relationships among the members of the Balansieae (Clavicipitales). *In* "Biology of Endophytic Microorganisms" (C. W. Bacon and J. F. White, Jr., Eds.), pp. 3–20. CRS Press, Boca Raton, FL.

Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* **51,** 221–271.

Yates, I. E., Hiett, K. L., Kapezynski, D. R., Smart, W., Glenn, A. E., Hinton, D. M., Bacon, C. W., Meinersmann, R., Liu, S., and Jaworski, A. J. 1999. GUS transformation of the maize fungal endophyte *Fusarium moniliforme*. *Mycol. Res.* **103,** 129–136.